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Immunomodulatory Effects of Poly(I,C)-LC in Cancer Patients

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Summary: Poly(I,C)-LC was administered in low (1 mg/m²) and intermediate (4 mg/m²) doses to cancer patients by intramuscular injection or intravenous infusion to evaluate the immunomodulatory effects. Natural killer cell (NK) activity was elevated slightly at the low dose and remained unchanged overall, but some depression was observed at the 4 mg/m² intravenous dose. Monocyte function was elevated in all groups of patients, as was the interferon-induced enzyme 2′5′-oligo-A synthetase. These increases were observed at the 1 mg/m² intramuscular dose, despite a lack of detectable circulating serum interferon (IFN). In regard to cell surface markers, poly(I,C)-LC induced an increase in OKT10-positive cells and a small but consistent trend toward increases in the ratio of Leu-3/Leu-2-positive cells. Lymphocyte proliferation in response to concanavalin A was depressed by poly(I,C)-LC administration. Although an optimum immunomodulatory dose and schedule was not determined, the data indicate that low doses produce significant changes in immune function and that induction of detectable levels of circulating interferon is not required for poly(I,C)-LC to have biological effects. **Key Words:** Interferon—Natural killer cells—Poly(I,C)-LC.

Polyinosinic-polycytidylic acid [poly(I,C)] is a synthetic, double-stranded nucleic acid polymer that is an effective inducer of interferon (IFN) in rodents (1). It is not, however, efficient in inducing IFN production in primates (2). Studies revealed that this was due to the presence of serum ribonucleases, which are capable of hydrolyzing poly(I,C) and are present in higher concentrations in primate sera than in rodent sera. A formulation of poly(I,C) complexed with poly-L-lysine and carboxymethylcellulose [poly(I,C)-LC] is relatively resistant to hydrolysis by primate sera and is capable of inducing IFN production in both monkeys and humans (3).

In addition to its properties as an IFN inducer, poly(I,C)-LC has many effects on the immune system. The activity of natural killer (NK) cells and macrophage function

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have been shown to be significantly increased (4), and significant antitumor effects in a rodent model have been observed (5). Several clinical trials have been performed, and the results are summarized elsewhere in this volume. For the most part, these trials have been designed to determine clinical toxicity and antitumor effects. In the studies presented here, the primary objective was to determine the immunomodulatory properties of poly(I,C)-LC when given to cancer patients at low and intermediate doses. The clinical aspects of the trial are summarized elsewhere in this volume by Stevenson et al.

MATERIALS AND METHODS

Study Outline

Fifty-nine patients with a variety of malignancies were treated with low (1 mg/m^2) or intermediate (4 mg/m^2) doses of poly(I,C)-LC and were monitored for changes in a number of immunological functions. Twenty patients received poly(I,C)-LC by intramuscular injection twice weekly (12 patients received 1 mg/m^2; 8 patients received 4 mg/m^2). Seventeen patients received poly(I,C)-LC once weekly by 1-h intravenous infusion (9 patients, 1 mg/m^2; 8 patients, 4 mg/m^2), and 22 patients received twice weekly poly(I,C)-LC by 1-h intravenous infusion (15 patients, 1 mg/m^2; 7 patients, 4 mg/m^2). Blood specimens for immunological monitoring were obtained 2 or 3 times prior to starting poly(I,C)-LC (day 0) and at days 1, 3, 7, 8, 10, 14, 15, 17, 21, 23, and 24, i.e., prior to and 24 and 72 h following injections of poly(I,C)-LC. Not all samples were available from all patients.

Preparation of Effector Cells

Mononuclear cells were obtained from heparinized blood by Ficoll-Hypaque separation. Fresh cells were used in the NK assay. The other assays were performed on cryopreserved cells as previously described (6).

Assays

Natural cytotoxicity was measured in a standard 4-h ^{51}Cr-release assay against K562 (7).

Monocyte function was measured in a growth inhibition assay (GIA), which assesses the ability of the monocytes and other mononuclear effector cells to inhibit the growth of an NK-resistant target cell line (MBL-2). The assay was performed in Terasaki plates using 10 μl of target cells (5×10^4/ml) and 10 μl of peripheral blood mononuclear cells at effector-to-target (E:T) ratios of 90:1, 30:1, 10:1, and 3:1 (7). The growth of the cells in each group was compared with that in the control wells, to which no effector cells were added.

Lymphoproliferative responses to concanavalin A (Con A) and mixed allogeneic leukocytes (MLC) were performed in a miniaturized version of a standard assay (8).

2′5′-oligo-A (2′5′A) Synthetase, an interferon-inducible enzyme, was measured by a competitive immunoenzymometric assay as previously described (9).

Dilutions of each extract were tested for their ability to inhibit the binding of a monoclonal antibody (3AC9) directed against 2′5′-A to microtiter plates precoated with a conjugate of p5′A2′p5′A2′p5′ and AECM-Ficoll.

Enumeration of Leukocyte Subpopulations

Enumeration of leukocyte subpopulations was performed by using a panel of monoclonal antibodies directed against cell surface antigens and detection using flow cytometry. The antibodies used in this study and their specificities were: anti-Leu-4, T lymphocytes; anti-Leu-3a + 3b, T helper and inducer lymphocytes; anti-Leu-2a, suppressor and cytotoxic T cells and some large granular lymphocytes, anti-Leu-11, Fcγ receptor on large granular lymphocytes, which are closely associated with NK activity; anti-Leu-12, B lymphocytes; OKT10, large granular lymphocytes and activated T cells; anti-Leu-M3, monocytes; and mouse IgG_1 and mouse IgG_{2a+2b}, negative controls. Unconjugated and FITC-conjugated human IgG was purchased from Cappel Laboratories, West Chester, PA, and FITC-conjugated goat $F(ab)_2$ anti-mouse IgG was purchased from TAGO, Inc., Burlingame, CA. Prepared samples were analyzed on an Ortho Cytofluorograf System 30-H, with a 2150 computer (Ortho Diagnostics Systems, Westwood, MA, U.S.A.). Dead cells were excluded from analysis by red fluorescence, i.e., staining of the DNA in dead cells by propidium iodide.

Fluorescence determinations for the lymphocyte and NK monoclonal reagents were performed by gating on the lymphocyte population only, while fluorescence determinations for the monocyte monoclonal, Leu-M3, were based on total peripheral blood mononuclear cells (PBMC).

Standardization of Assays

To reduce the problem of assay variation, studies of growth inhibitory function, lymphocyte proliferation, 2′5′A synthetase, and surface markers were performed on cyropreserved cells from the patients using the methods previously described (10).

For the NK assay, earlier studies in our laboratory (10) have shown that NK function of cells from some donors was not reliably cryopreserved. Because of this concern, the NK activity was always tested on fresh cells. A set of cryopreserved standards was used to normalize the patient data, as previously described (10).

Analysis of Change in Immune Function

It has been shown previously in our laboratory (10) that normal, untreated individuals show considerable variation in NK activity when tested repeatedly over a 28-day period. Because of this variability, an inaccurate assessment of any change may be made unless several pretreatment values and an estimate of this variability are obtained. The limits of variability for each individual were calculated as the mean of the pretreatment values ± 15% cytotoxicity. The procedure and calculations are discussed in detail elsewhere (10).

For the GIA, the limits of variability were set as the mean of pretreatment values ± 10% growth inhibition. The limits of variability for the lymphocyte proliferation responses were defined as the mean of the pretreatment values ± 1.6 SD. Using these criteria, values above or below the limits of variability were considered to represent increases or decreases in activity that were likely to be the results of poly(I,C)-LC administration.

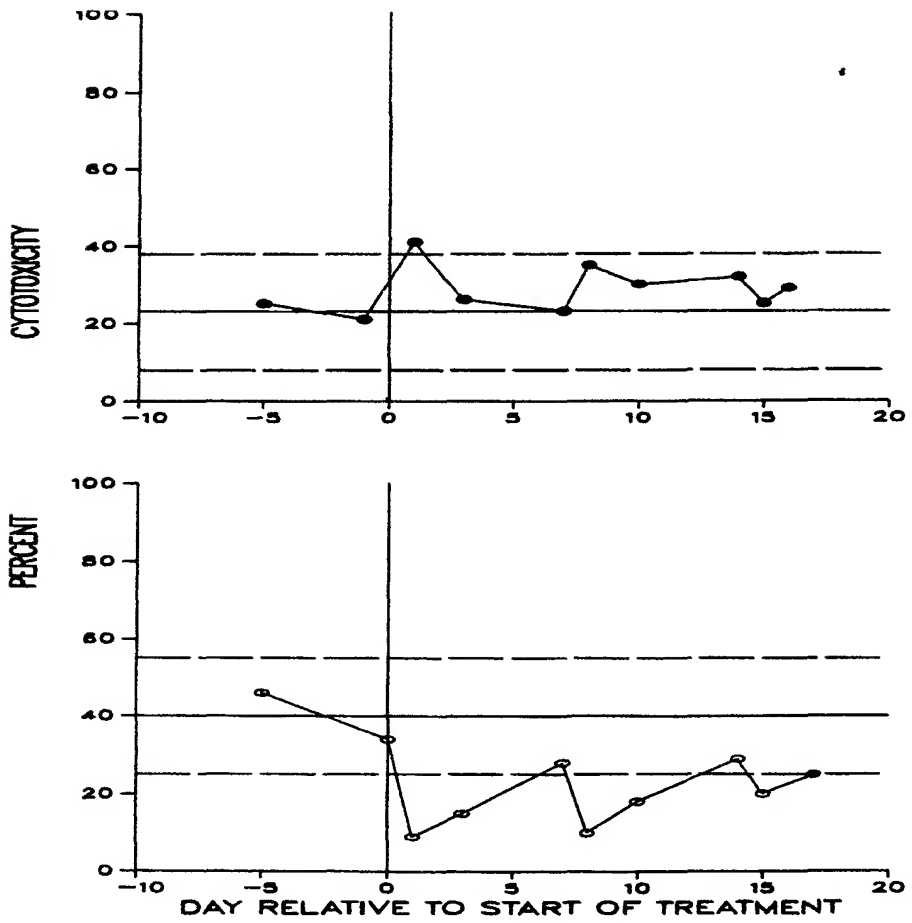

FIG. 1. NK activity at effector-to-target ratio of 90:1 for two patients who received poly(I,C)-LC 1 mg/m² (filled circles) or 4 mg/m² (open circles) weekly by intravenous infusion on days 0, 7, and 14. Data are expressed as percent cytotoxicity. The dotted horizontal lines indicate the limits of variability, defined as mean pretreatment value (solid horizontal line) ± 15% cytotoxicity.

RESULTS

Analysis of Change of NK Activity

A graphic representation of NK activity for two patients is shown in Fig. 1. The limits of variability, as defined above, are shown as horizontal dotted lines. One patient (top panel) received 1 mg/m² poly(I,C)-LC once weekly by intravenous infusion and only one change outside the limits of variability was observed. The other patient (lower panel) received 4 mg/m² intravenously once weekly, and a majority of values fell below those defined limits. An analysis of the data for all patients to determine the change outside the limits of variability is presented in Table 1. Although the majority of values for all doses and schedules fell within the predetermined limits, 11% of values were increased and only 3% were decreased on the 1 mg/m² intra-

TABLE 1. *Analysis of change in NK activity following poly(I,C)-LC*

Protocol	Dose	Changes outside variability limits (% of values)		
		Increase	Decrease	No change
i.m. 2/wk	1 mg/m²	11	3	86
i.m. 2/wk	4 mg/m²	6	17	77
i.v. 1/wk	1 mg/m²	3	14	83
i.v. 1/wk	4 mg/m²	8	38	54
i.v. 2/wk	1 mg/m²	7	14	79
i.v. 2/wk	4 mg/m²	0	37	63

NK, natural killer; i.m., intramuscular; i.v., intravenous.

muscular schedule. In contrast, 4 mg/m² given intravenously, either once or twice each week, resulted in 38 and 37% of the values being decreased, respectively.

Analysis of Change in GIA and Lymphocyte Function

In contrast to the infrequent alterations in NK activity, GIA in all treatment groups was increased rather consistently, with >50% of the values being above the limits of variability.

Lymphocyte proliferation in response to concanavalin A was uniformly low in patients prior to treatment. Treatment with poly(I,C)-LC resulted in significantly further depression of the proliferative response.

Changes in 2'5'A Synthetase Activity

The 4 mg/m² intravenous schedule resulted in significant levels of serum IFN, whereas on the 1 mg/m² intramuscular schedule, no circulating IFN was detectable. Levels of 2'5'A produced in normal PBMC were 20–80 pmol 2'5'A/h/10^6 cells. Highly significant elevations in 2'5'A were observed in all patients tested, from all treatment groups. In Fig. 2, the 2'5'A activity in cells from a representative patient who received 1 mg/m² poly(I,C)-LC by intramuscular injection is presented.

Change in Leukocyte Subpopulations

Using a panel of monoclonal antibodies directed against cell surface markers, the number of positive cells at various times before and during poly(I,C)-LC treatment was determined. No significant changes were observed in the percentage of total T lymphocytes or B lymphocytes. There was a small but consistent elevation in the ratio of Leu-3 (helper/inducer) to Leu-2 (suppressor/cytotoxic) subpopulations of T lymphocytes. This was caused by an increase in the number of Leu-3-positive cells and a decrease in the number of Leu-2-positive cells. Although these changes were not significant for any one patient, the trend was consistent. OKT10, which is expressed in large granular lymphocytes and activated T and B lymphocytes, was found to increase consistently in all treatment groups. In contrast, a slight decrease was found in the number of cells expressing Leu-11, an antigen found on large granular lymphocytes that have been shown to mediate NK activity. This decrease was not significant for any patient, but the trend was consistently observed.

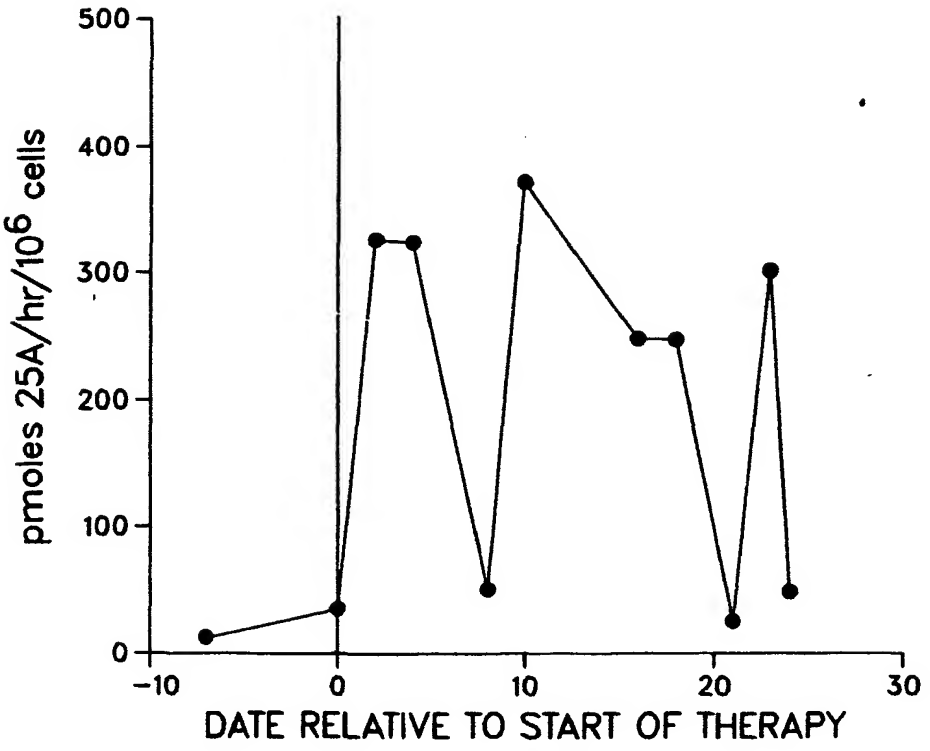

FIG. 2. 2′5′A activity expressed as pmol synthesized/h/10^6 cells in PBMC from a patient who received poly(I,C)-LC 1 mg/m² by intramuscular injection on days 0, 4, 7, 10, 14, 17, 21, and 24.

DISCUSSION

Poly(I,C)-LC was shown to be immunomodulatory when given to cancer patients at low or intermediate doses. This treatment resulted in increased growth inhibitory activity, increased levels of 2′5′A synthetase, increased percentages of OKT10-positive cells, and decreased lymphoproliferative responses.

Poly(I,C)-LC has been viewed mainly as an IFN inducer. At the 4 mg/m² intravenous dose, significant levels of IFN (300 U) were detectable in the serum. At the lower intravenous dose, less IFN was produced. In contrast, 1 mg/m² of poly(I,C)-LC by intramuscular injection induced no detectable levels of IFN in the serum. However, levels of 2′5′A synthetase, an IFN-induced enzyme, were elevated in PBMC of all poly(I,C)-LC-treated patients, even in the absence of detectable levels of circulating IFN. These increases in 2′5′A synthetase may reflect production of IFN in PBMC or their exposure to low levels of IFN in the plasma or at extramuscular sites. Thus, it is unclear whether the immunomodulatory effects of poly(I,C)-LC are all attributable to the induction of IFN or whether this agent also has other, non–IFN-dependent effects on the immune system.

The augmentation of growth inhibitory activity by PBMC was the most consistent positive functional effect of the poly(I,C)-LC treatment. This antitumor cytostasis activity is mediated by monocytes in untreated normal donors or cancer patients, but it remains unclear whether or not other effector cells also contribute to the augmented

reactivity upon poly(I,C)-LC treatment. As strong augmentation of this activity was observed in patients receiving the low dose of poly(I,C)-LC, either intramuscularly or intravenously, it will be of interest to determine the minimal dose required to affect this potentially important effector mechanism.

In contrast to the consistent augmentation of GIA, overall NK activity was unchanged. In fact, at the higher intravenous dose of 4 mg/m^2, NK activity was frequently depressed. This overall lack of augmentation of NK activity is in contrast to the consistent ability of poly(I,C)-LC in vitro to augment NK activity of normal donors and cancer patients. The lack of in vivo augmentation and the paradoxical depression of NK activity at higher dose levels of poly(I,C)-LC are reminiscent of the pattern of results previously obtained in trials with patients receiving IFN-α (10). As in the IFN trials, the highest percentage of augmented NK values was seen in patients receiving the low-dose intramuscular treatment.

The effects of poly(I,C)-LC treatment on cell surface markers were also similar to those induced by IFN (7). The main increase in marker expression was the higher percentage of OKT10-positive cells. As the relative proportions of T cells and large granular lymphocytes seemed to be unchanged, this increase in OKT10 expression was probably an indication of activation of some of the PBMC.

Treatment with poly(I,C)-LC also had a noticeable effect on lymphoproliferative responses. Although the baseline proliferative responses of the cancer patients were below normal, poly(I,C)-LC induced a further depression. However, this inhibitory effect of poly(I,C)-LC was not as great as that seen in the IFN-α trials (7), which was perhaps related to the lower doses of circulating IFN.

Further Phase I trials will be needed to determine the optimal immunomodulatory dose and schedule of administration of poly(I,C)-LC. From the present results, it appears that the low dose of poly(I,C)-LC, administered intramuscularly, was associated with the greatest augmentation of NK activity, levels of augmentation of growth inhibitory activity similar to those induced by the higher dose, clear elevations in 2'5'A synthetase, and the least depression of lymphoproliferative responses. Such results are encouraging, as they indicate that substantial immunomodulation by poly(I,C)-LC can be induced by relatively nontoxic levels of the agent. It will be important to evaluate the effects of even lower doses of poly(I,C)-LC than those tested in the present trial so as to determine whether or not a dose and schedule can be achieved that will induce sustained elevations in NK activity, as well as in growth inhibitory activity, in the absence of appreciable inhibition of lymphoproliferative responses. Such an optimal immunomodulatory schedule may have greater antitumor efficacy than the schedules studied to date.

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